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A. PROGRESS REPORT

THERAPEUTIC PROPERTIES OF BLOOD PLASMA IN THE
THERAPY OF TOURNIQUET SHOCK

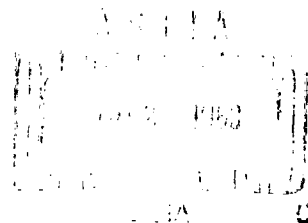
1 January 1962 to 31 December 1962

Morton D. Pareira, M.D., Responsible Investigator
The Jewish Hospital of St. Louis
216 South Kingshighway Boulevard
St. Louis 10, Missouri

Department of Defense Contract DA-49-007-MD-799

Supported by: Research and Development Division
Office of the Surgeon General
Department of the Army
Washington 25, D. C.

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ABSTRACT

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216 South Kingshighway
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3. Principal Investigator: Morton D. Pareira, M.D.
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4. Reconstituted human plasma is significantly more effective in the therapy of rat tourniquet trauma than human albumin when both are adjusted to the same total protein concentration. This observation questions whether the resuscitative effect of the human plasma is due to its oncotic effect only or to some property of the plasma present in the non albumin fraction.

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Body of the Report

During 1961 (see Progress Report 1 January to 31 December 1961) this laboratory attempted an investigation of the therapeutic properties of rat tourniquet convalescent plasma in the therapy of rat tourniquet trauma. During that study it was noted that reconstituted lyophilized human plasma was strikingly more effective than any of the rat plasmas which we had prepared, whether normal or convalescent. Because the rat plasmas had been collected under non-sterile conditions they were all refrigerated to inhibit bacterial growth. Both of these factors were considered possible reasons for a loss of therapeutic efficacy in the rat plasma. The possibility was also considered that the aging or the processing of the human lyophilized material had in some way converted it into a more effective resuscitative solution. Preliminary studies of the effect of liquid, shelf stored human plasma showed it to be as effective as the lyophilized material (see Application for Research Contract for the current year).

The factors involved in adequate preparation of rat plasma and the improved performance of the human lyophilized material have been investigated in the following manner.

- (1) A method for the sterile preparation of rat plasma was sought.
- (2) Attention was devoted to the factors of aging, freezing, and protein concentration in the preparation of rat plasma. An analysis of electrophoretic patterns in these different plasmas was carried out.
- (3) An attempt was made to separate the various factors which might be involved in the therapeutic properties of human plasmas prepared in different ways. Attention was devoted to pooling, aging, lyophilization, total protein concentration, albumin content, and analysis of electrophoretic patterns.

These studies will be described in detail in the remainder of the report.

Methods

Tourniquet Trauma

Male albino rats of the Holtzman Farms, weighing 200-280 grams, were used. Each animal was individually housed in an air-conditioned laboratory for at least four days before use. Food was removed from the cages approximately 15 hours before the application of tourniquets. On the morning of the

experiment all animals were weighed, anesthetized and bilateral hind limb tourniquets applied for 4-1/2 hours. (This duration of tourniquet trauma has been previously shown to be lethal to at least 96% of untreated controls in this weight range.)

At the time of tourniquet release, the animals were again anesthetized and a polyethylene catheter introduced into the right jugular vein for administration of the therapeutic solution.

The infusion was begun one hour after the release of tourniquets and the rate of infusion so timed that the solution was administered over a period of 3 hours \pm 9%. The infusion fluid contained 500 u penicillin per ml. Infusions were given simultaneously by means of 2 or 3 variable speed constant infusion pumps.

The vein was ligated at the completion of the infusion. After skin closure the animals were returned to their cages where food and water were available to them. Survival was recorded at 24 and 48 hours after tourniquet release.

Preparation of Sterile Plasma

The first approach was made by using various antibacterial agents (merthiolate, penicillin, streptomycin, chloromycetin, etc.). Some of these were placed into the plasma after collection, some were administered to the animals prior to bleeding. The possibility of alteration of protein properties or that these materials might have toxic properties in shocked animals made all of these preliminary approaches undesirable in principle. Accordingly we were enthusiastic about the possibility that the millipore filter system might be used to solve this problem. This system has, in fact, provided a reliable means of preparation of sterile plasma after non-sterile collection of blood from rats. It has also been used to prepare sterile human plasma for shelf storage.

In essence a non-sterile liquid is passed through a series of non-sterile filters of decreasing pore size. The final filter has a pore size of 0.22 micra, which is smaller than the smallest bacterium. The final passage is made into a pre-sterilized bottle after autoclaving the filter equipment. The sterile bottled product may then be kept at the desired temperature for any length of time. Bacteriologic study of the plasma prior to use in each instance proved it to be sterile. A comparison of the millipore filtered lyophilized plasma after reconstitution with its unfiltered counterpart showed no difference in therapeutic effectiveness or electrophoretic pattern.

Studies of Rat Plasmas

Effect of Freezing

It will be recalled that preliminary observations made in the 1 January to 31 December 1961 Progress Report suggested that freezing of rat plasma might diminish its therapeutic properties significantly.

Two experiments were therefore performed during the current period. In one, prior to the use of millipore sterilization, the rat plasma was collected in a non-sterile manner and then quickly frozen. On the day of the experiment it was thawed and filtered (blood set filter) and administered to half the animals. The other half was given plasma collected in a non-sterile manner just prior to administration. Penicillin was added to both plasmas in the usual way prior to administration. Total plasma protein concentration was 4.6 grams per cent in each.

Comparison of Recently Frozen and Just Collected Non-Sterile Rat Plasma

Infusion (Volume Equal to 3 Per Cent Body Weight)

	48 Hour Survival	
	<u>Lived</u>	<u>Died</u>
Fresh Plasma	9	9
Frozen Plasma	9	9

This experiment was repeated later in the year using the millipore filter system to prepare sterile plasma. In these experiments one batch of rat plasma was collected about 5 days before use, sterilized, and then frozen in the deep freeze (-20°C). On the afternoon prior to the experiment it was placed in the refrigerator for slow rewarming. On the day of the experiment it was thawed to room temperature, slowly filtered in a standard blood set filter, penicillin added, and the plasma infused.

The "fresh" material was collected and processed the day before infusion, then shelf stored till the following day when penicillin was added just prior to infusion.

Comparison of Rat Plasmas in the Therapy of Tourniquet Trauma

Infusion (Volume Equal to 4 Per Cent Body Weight)

	48 Hour Survival	
	<u>Lived</u>	<u>Died</u>
Fresh Plasma	0	15
Frozen Plasma	2	13

	<u>Na</u>	<u>K</u>	<u>Cl</u>	<u>Total Proteins</u>
Fresh Plasma	139	3.2	88	5.3
Frozen Plasma	140	3.2	88	5.2

In these two experiments freezing did not alter the therapeutic properties of the plasma. As will be shown in the section on electrophoretic studies, there do seem to be alterations of the protein components of the plasma induced by freezing.

Effect of Aging

In this experiment, rat plasma (after collection in 1 part ACD: 5 parts plasma) was passed through the millipore sequence into a sterile flask and stored for 5 months at room temperature. On the day before the experiment an additional lot of rat plasma was collected, sterilized and kept overnight at room temperature.

On the day of the experiment, penicillin was added to each and the infusion given. The total protein concentration of the aged plasma was diluted from 5.2 to 4.6 mgm% with sterile saline. The fresh plasma total protein concentration was 4.5 mgm%.

Comparison of Aged and Fresh Rat Plasma
in Tourniquet Trauma

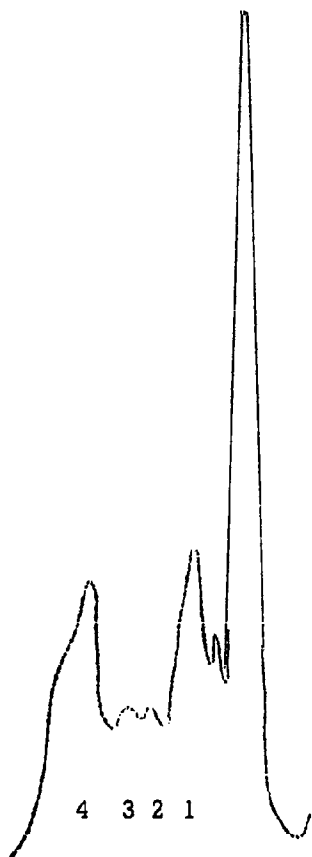
Infusion (Volume Equal to 3 Per Cent Body Weight)

	48 Hour Survival	
	<u>Lived</u>	<u>Died</u>
Fresh Plasma	4	14
Aged Plasma	1	17

These results showing no improvement of rat plasma with aging are in striking contrast with the results obtained with human plasma. These will be described subsequently.

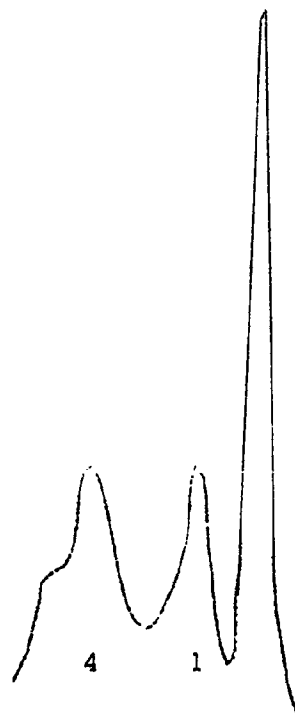
Rat Plasma Electrophoresis (Figs. 1, 2, 3, 4)

Preliminary studies show that the peaks labeled 2 and 3 which are present in fresh rat plasma are altered in aged and frozen plasma. In the frozen plasma the peak labeled 2 is absent; in the aged plasma both 2 and 3 are absent. Since our studies to date show no difference in therapeutic effectiveness between these different types of plasmas, these alterations do not seem important in this area of primary interest.



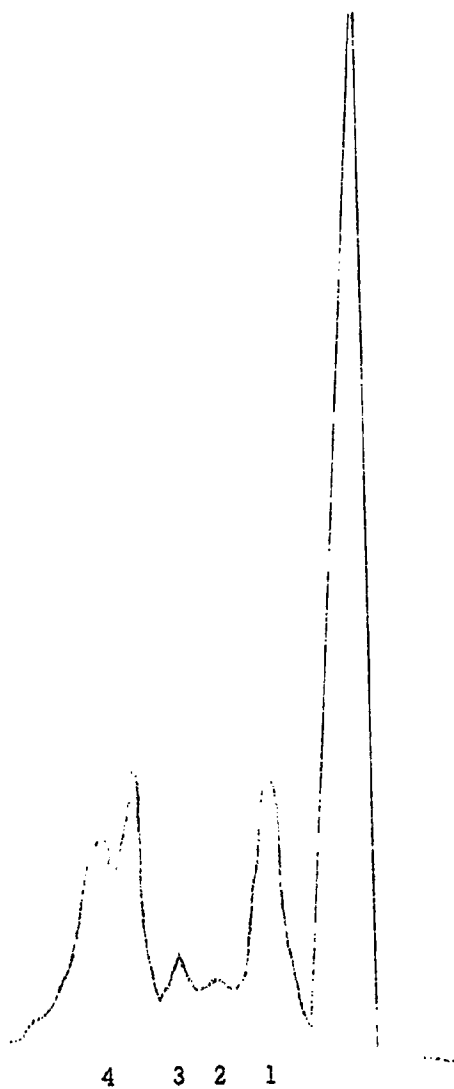
Rat Plasma, Fresh

Figure 1



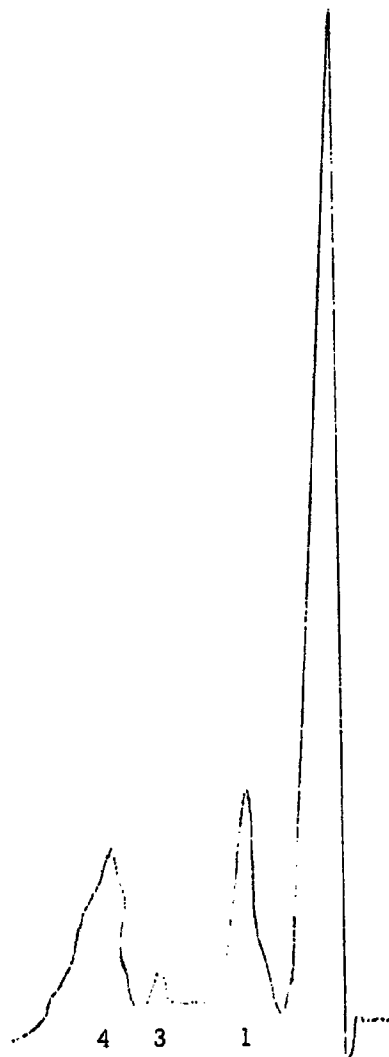
Rat Plasma, Shelf Stored

Figure 2



Fresh Rat Plasma

Figure 3



Frozen Rat Plasma

Figure 4

Experiments and Results

Studies of Human Plasmas

In the first group of experiments, simultaneous comparison was made after the standard tourniquet trauma of (1) "fresh" (just collected) plasma from a series of single donors, (2) pooled plasma shelf stored in the blood bank at room temperature for six months and (3) reconstituted, lyophilized plasma obtained commercially from the Hyland Laboratories. Each of the three solutions was infused into a group of 12 animals at a time; a single day thus allowed 36 simultaneous infusions. The experiment was conducted on three different days.

Comparison of Human Plasmas in the Therapy of Tourniquet Trauma in the Rat

Infusion (Volume Equal to 3 Per Cent Body Weight)

	48 Hours after Survival	
	<u>Lived</u>	<u>Died</u>
Fresh (individual donor)	0	35
Shelf stored (pooled, 6 months at room temperature)	24	11
Reconstituted, lyophilized (pooled, commercial)	26	8

These data were interpreted to show that either pooling or aging had greatly improved the therapeutic effectiveness of the plasma. Since the shelf stored liquid plasma was as effective as the lyophilized material the lyophilization process was ruled out as a cause of improvement in survival results. Whether the improvement was based upon removal of something harmful from the fresh plasma or the addition of a beneficial substance with aging or pooling has not been determined.

For each type of plasma, rat cells were crossmatched against donor plasma and showed clumping of cells. During one experiment one animal from each group was sacrificed to allow inspection of the serum for hemolysis. In the

fresh and shelf stored animals hemolysis was present to a slight degree. In the animal infused with the reconstituted lyophilized plasma a "moderate" degree of hemolysis was present. It would thus seem that although some hemolysis was present in all groups, this did not interfere with the beneficial therapeutic effect. Other observations would suggest that (at least) some of this hemolysis is due to the trauma rather than the infusion material.

The second group of experiments was designed to show whether pooling alone improved therapeutic effectiveness and, when it did not, what happened to therapeutic effect with aging of pooled plasma. In these experiments human plasma from six donors was collected, and processed through the millipore filter sequence into aliquots. The aliquots were used in the following series of experiments to compare different plasmas in the therapy of the standard tourniquet trauma.

- (1) fresh pooled plasma (6 donors) vs. fresh plasma from a single donor
- (2) 1 week old pooled plasma vs. lyophilized plasma
- (3) 5 week old pooled plasma vs. lyophilized plasma
- (4) 13 week old pooled plasma vs. lyophilized plasma
- (5) 14 week old pooled plasma vs. lyophilized plasma

Throughout this sequence of experiments, the protein concentration of solutions to be infused was determined by use of a Total Solids optical density meter. Discrepancies in protein concentration of solutions on any given day were equalized by dilution with 0.9% sodium chloride solution.

Comparison of Human Plasmas in the Therapy of Tourniquet Trauma

Infusion (Volume Equal to 3 Per Cent Body Weight)

	48 Hour Survival	
	<u>Lived</u>	<u>Died</u>
Fresh, pooled plasma	0	18
Fresh, single donor plasma	0	17

Thus, it was demonstrated that pooling was not the factor responsible for improvement in results.

Comparison of Human Plasmas in the Therapy of Tourniquet Trauma

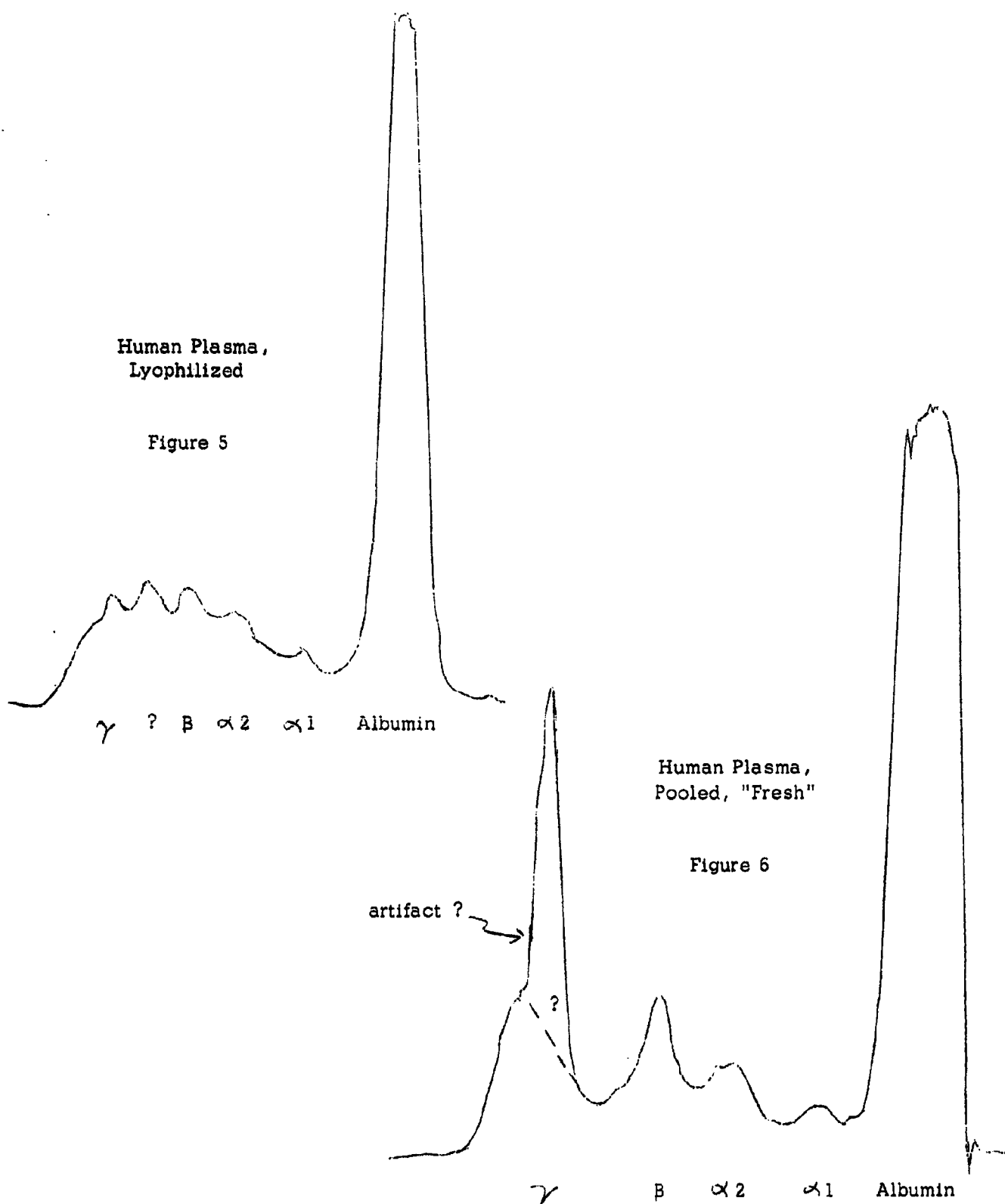
Infusion (Volume Equal to 3 Per Cent Body Weight)

	48 Hour Survival	
	<u>Lived</u>	<u>Died</u>
Pooled, 1 week old	1	17
Lyophilized, Reconstituted	16	0
Pooled, 5 weeks old	8	9
Lyophilized, Reconstituted	11	7
Pooled, 3 months old	22	14
Lyophilized, Reconstituted	22	13

It was apparent that the aging process alone in some way converted a non-effective or deleterious solution into one which was highly effective in the treatment of this trauma.

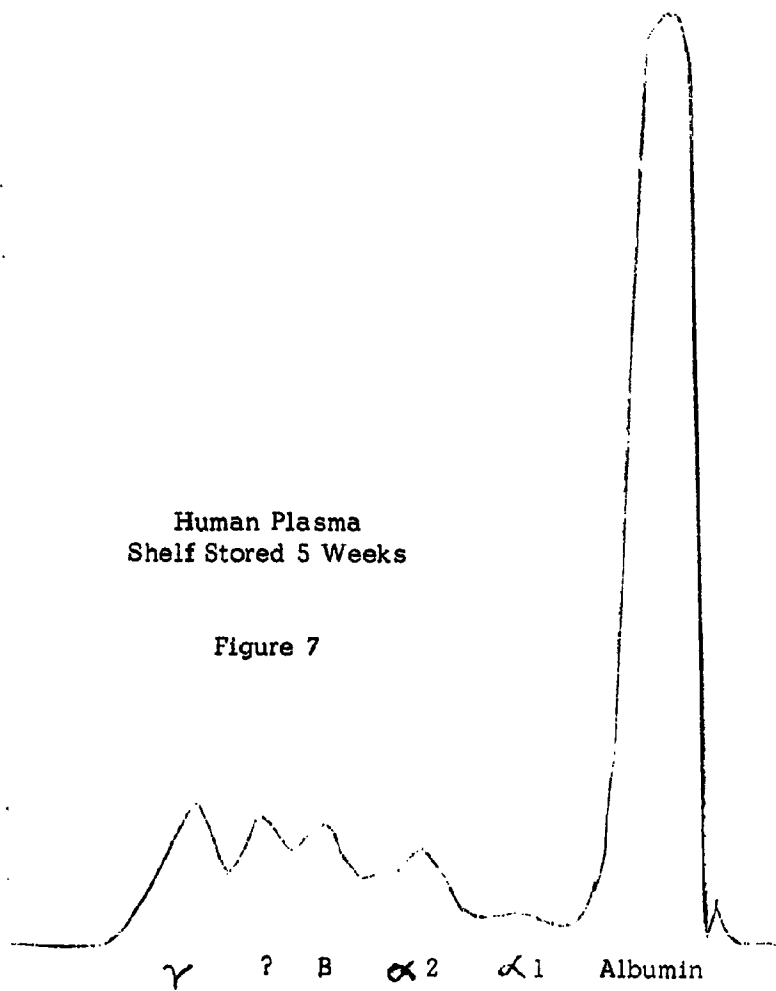
Sequential electrophoretic studies on these plasmas revealed the following patterns. Note in the fresh pooled plasma (Fig. 6) there are 4 peaks to the left of the albumin peak while in the lyophilized plasma (Fig. 5) there are 5. The height of the gamma globulin peak in the fresh pooled plasma we believe is artifactual due to precipitated protein on the strip. However, there is a peak present between the beta and gamma globulins in the lyophilized pattern which is clearly not present in the fresh. When the electrophoresis patterns of the aged aliquots were run at the time of their use (Figs. 7 and 8), the patterns revealed that each of them contained the intermediate peak between the original beta and gamma peaks. A precise quantitative measurement of the location of these peaks is not shown. We have improved our techniques for electrophoretic analysis and intend to repeat this study using these more discriminating techniques with precise measurement of peak location.

The final studies of properties of human plasma were devoted to testing the importance of total protein concentration and albumin content. In each of two experiments, one group was infused with a 5% human albumin solution (Albumisol, Merck Sharp and Dohme) while another group was infused with



Human Plasma
Shelf Stored 5 Weeks

Figure 7



Human Plasma, Shelf Stored, 3 Months

Figure 8

reconstituted lyophilized plasma diluted with 0.9% sodium chloride solution so that the total protein concentration was about the same in both solutions. In the second experiment, electrolyte concentrations were also determined.

Comparison of Albumisol and Lyophilized Plasma
Infusion (Volume Equal to 3 Per Cent Body Weight)

	48 Hour Survival	
	Lived	Died
Albumisol	3	15
	<u>7</u>	<u>11</u>
	10	26
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Lyophilized Plasma	14	3
	<u>10</u>	<u>7</u>
	24	10
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	Electrolytes (Meq/L)			Total Protein Concentration (gms%)
	Na	K	Cl	
Albumisol	151.5	0.75	121	4.9
Lyophilized Plasma	183.0	4.0	115	4.5

These figures are startling; we intend to repeat this experiment with certain variations to be certain of the reproducibility of the observation. As it stands it would seem that the Albumisol solution, which represents a colloid-electrolyte solution every bit as good as plasma, is far less effective at this dose level than plasma in the therapy of this trauma. This observation brings to mind the results of previous experiments (1) in which dextran-in-saline was found to be only as effective as saline solution even though it is also both a colloid and electrolyte solution with dextran particle size about the same molecular weight as albumin. It is said that about 80% of the oncotic pressure exerted by plasma is due to its albumin content. The lyophilized

plasma with its total protein concentration adjusted to that of the albumin therefore should have a less potent oncotic effect than the albumisol. The conclusion must be that there is present in the non-albumin portion of the plasma some factor highly effective in the therapy of this trauma. In future experiments, electrolytes will be adjusted to provide equal concentrations in both solutions.

Recapitulation

- (1) Utilization of the millipore filter system has made possible the preparation of sterile plasma eliminating the need for the addition of antibiotics or chemical antibacterial agents.
- (2) Aged rat plasma is no more effective in the therapy of rat tourniquet trauma than freshly collected rat plasma. Frozen rat plasma also seems to be as effective as freshly collected plasma. None of the rat plasma preparations are as effective as aged or lyophilized human plasma.
- (3) Recently collected single donor or pooled human plasma allows no survival when infused after the standard rat tourniquet trauma. Shelf-stored, pooled human plasma (6 months at room temperature) is as effective as reconstituted lyophilized human plasma (highly effective) in the therapy of this trauma. Pooled human plasma, as it ages, gradually acquires the ability to protect, so that, by three months after collection, it is as effective as the commercial lyophilized material. At the time significant protection is first achieved, electrophoretic studies show the appearance of a band not present in the fresh material. This band is also present in the lyophilized reconstituted plasma.
- (4) Reconstituted human plasma is significantly more effective in the therapy of rat tourniquet trauma than human albumin at the dose level tested when both are adjusted to the same total protein concentration. This observation makes highly questionable the assumptions that the resuscitative effect of the human plasma is due to its oncotic effect only. The properties present in the non-albumin fraction would also seem to have an important bearing on the resuscitative properties of the plasma.
- (5) Sections IV and V under Methods of Research in the current Application for Research Contract have not as yet been pursued. Section IV, a comparison of serum vs. plasma, will be undertaken shortly. Section V, the evaluation of rat tourniquet convalescent serum or plasma will be postponed until the current studies of human plasma have been followed as far as seems indicated.

Implications

- (1) The use of the tourniquet traumatized rat as a bio-assay device for measuring therapeutic effectiveness of different resuscitative solutions would seem to have significant potential value at the investigative level and perhaps eventually in clinical studies as well.

That aged human plasma has different properties than fresh, that this kind of human plasma is more effective than rat plasma for this trauma, and also more effective than its albumin-electrolyte equivalent suggests that there is a specific protective factor in the non-albumin portion of aged human plasma.

The possibilities of identifying the cause of improvement in aged human plasma, and of being able to isolate a specific protective fraction by protein separation devices make this, we believe, a fruitful area to pursue.

Publications

1. Enhanced Efficacy of Plasma after Aging in the Treatment of Tourniquet Shock. Serkes, K. D., Lang, S. and Pareira, M. D. Surgical Forum, 13: 12, 1962.
2. No. 1 was presented in the Fundamental Forum on Shock at the Clinical Congress of the American College of Surgeons, October 16, 1962. The expanded manuscript of the presentation is now to be submitted for publication.

Bibliography

Efficacy of Plasma and Dextran Compared to Saline for Fluid Replacement Following Tourniquet Shock. Serkes, K. D., Lang, S. and Pareira, M. D. Surgery, 45: 623, 1959.